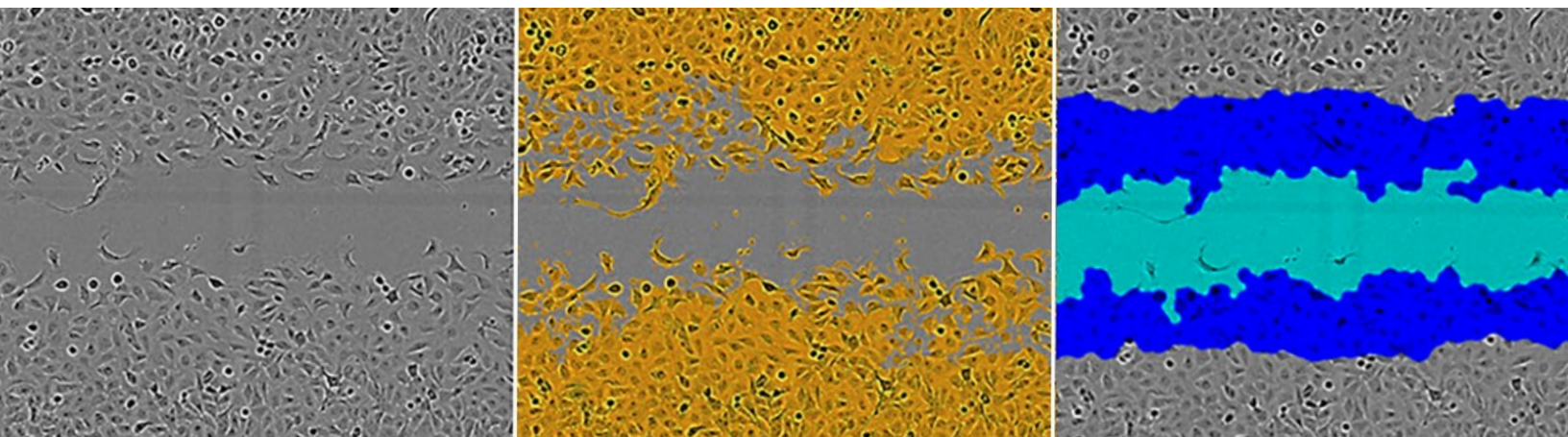




IncuCyte ZOOM® 96-Well Scratch Wound Cell Migration & Invasion Assays User Manual

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Welcome to your WoundMaker™

The WoundMaker™ is a 96-pin mechanical device designed to create homogeneous, 700-800µm wide wounds in cell monolayers on 96-well ImageLock™ microplates. The device is simple to use and wounds can be created in seconds.

When used, stored and cleaned correctly the WoundMaker™ should continue to provide clean, consistent wounds without damaging the cells or the underlying plastic or biomatrix. The WoundMaker™ is comprised of:



Getting started, Do's and Don'ts

1. Before using the WoundMaker™ for the first time, soak the pins in sterile water (45ml for 5min) and then sterilize with 70% ethanol (45ml for 5min).
2. **Treat the WoundMaker carefully at all times.** Never place the WoundMaker™ pin block directly on to the bench surface – this will damage the pins!
3. **Never** use the WoundMaker™ with a dry or partially dry plate. Make sure each well contains media or PBS before making a scratch.
4. **Never** use the WoundMaker™ with more than 100µl of media per well.
5. Do not push down on the WoundMaker™ pin block when making wounds.
6. Store the WoundMaker™ in a clean, dust free and safe location such as a biological safety cabinet when not in use.
7. Clean the WoundMaker™ pin block according to the wash protocol after every use.
8. Do not put more than 45ml of any solution into the wash boat. This will prevent damage to the pin mechanism caused by ingress of liquid into the pin block through capillary action.

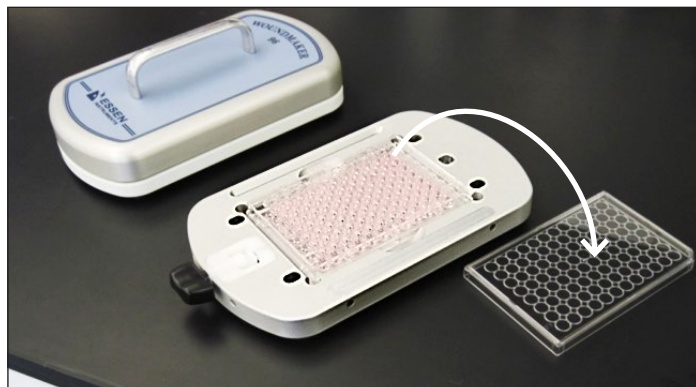
Creating wounds: Six simple steps

The following steps should be performed in a biological safety cabinet:



Step 1

- Remove top of WoundMaker™.
- Set top in empty wash boat.



Step 2

- Insert plate (containing cells & media) into base plate holder.
- **Remove plate cover.**



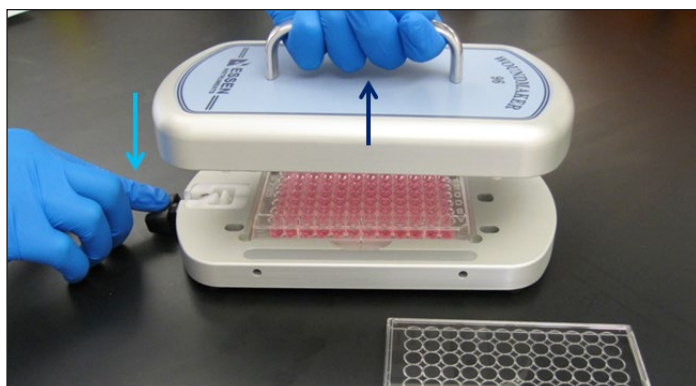
Step 3

- Replace pin block by guiding the rear dowels of pin block into the rear holes of the base plate.
- **Do not push down.**



Step 4

- Push and hold the black lever.



Step 5

- Lift pin block while continuing to hold the black lever down.



Step 6

- Replace plate cover.
- Wash wells (up to two washes).
- Add treatment conditions.
- Put into the IncuCyte™ and start acquiring data and images.

WoundMaker™ Wash Protocols & Storage

Prior to use after storage

- Fill wash boat 1 with 45 ml (no more, no less) of sterile distilled water and place WoundMaker™ pin block in the solution for 5 minutes. Do not agitate.
- Fill wash boat 2 with 45 ml (no more, no less) of 70% ethanol and place WoundMaker™ pin block in the solution for 5 minutes. Do not agitate.

When wounding multiple plates of the same cell line

- Between plates fill wash boat 1 with 45 ml (no more, no less) of sterile distilled water and place WoundMaker™ pin block in the solution for 5 minutes. Do not agitate.

After the last experiment of the day or when wounding plates of different cell lines

- Fill wash boat 1 with 45 ml (no more, no less) of 0.5% Alconox and place WoundMaker™ pin block in the solution for 5 minutes. Do not agitate.
- Fill wash boat 2 with 45 ml (no more, no less) of 1% Virkon S. and place WoundMaker™ pin block in the solution for 5 minutes. Do not agitate.
- Fill wash boat 1 with 45 ml (no more, no less) of sterile distilled water and place WoundMaker™ pin block in the solution for 5 minutes. Do not agitate.
- Fill wash boat 2 with 45 ml (no more, no less) of 70% ethanol and place WoundMaker™ pin block in the solution for 5 minutes. Do not agitate.
- Place the WoundMaker™ on its base plate and store it in a dust-free environment.

Sterilization using 70% ethanol for 5 min is sufficient to kill most pathogens. For information about the sensitivities of specific microbial agents please refer to CDC or other applicable agency:

http://www.cdc.gov/hicpac/disinfection_sterilization/6_0disinfection.html

Virkon®S is registered to DuPont Corporation. For more information regarding mode of action, composition and disposal, please visit:

<http://www.day-impex.co.uk/virkon-disinfectant.html>

Protocol Overviews

96-Well Scratch Wound Cell Migration Assays

Essen BioScience's IncuCyte™ 96-Well Scratch Wound Cell Migration assay is designed for fully automated, kinetic quantification of cell migration in vitro. There are 6 simple steps to assemble and run these assays:

1. Seed cells at an appropriate density (e.g. 20K cells well-1) into each well of a 96-well ImageLock™ tissue culture plate (Essen BioScience 4379) and incubate in a standard cell incubator for between 6 – 18h.
2. Remove the plate from the incubator, and use the WoundMaker™ and wounding procedure to create precise and reproducible wounds in all wells of the 96-well ImageLock™ plate.
3. After wounding, aspirate the media from each well and gently wash each well two times with culture media to prevent dislodged cells from settling and reattaching.
4. After washing, add 100 µL of media containing test material at the appropriate concentration (e.g. small molecules, antibodies).
5. Remove any bubbles from the assay plate.
6. Place assay plate into the IncuCyte ZOOM® and allow it to equilibrate for 5 minutes. Schedule repeat scanning (e.g. every 2-3h for 48h) in the ZOOM software: ensure 'scan type' is set to **Scratch Wound** and **Wide Mode** is selected if using 20x or 10x objectives.

An example protocol, where full details of each of these steps for a representative cell type are described, can be found in Section 5.

96-Well Scratch Wound Cell Invasion Assays

Essen BioScience's IncuCyte™ 96-Well Scratch Wound Cell Invasion assay is designed for fully automated, kinetic quantification of cell invasion. There are 9 simple steps to assemble and run these assays:

1. Coat a 96-well Essen ImageLock™ plate (Essen BioScience 4379) with a thin layer of biomatrix (e.g. collagen-1 or Corning® Matrigel® (Matrigel), 1 – 48h).
2. Seed cells at the optimized cell density (e.g. 20K cells well-1) into each well of the coated plate and incubate (37 C) in a standard cell incubator for between 6 – 18h.
3. Prepare biomatrix top layer (with or without test compounds, as required).
4. Carefully follow the 96-well WoundMaker™ procedure to create precise and reproducible wounds in all wells of the 96-well ImageLock™ plate. Wash cells with media to remove debris and to prevent dislodged cells from settling and reattaching.
5. Add 100 µl of media, then cool the cell plate to 4°C for 5' using the CoolSink and CoolBox 96F (1500-0080-A00).
6. Aspirate media, and overlay cells with biomatrix top layer (collagen-1 or Matrigel). Remove any bubbles from the assay plate.
7. To gel the top layer warm the cell plate to 37°C by placing onto a warm CoolSink (1500-0080-A00) in the cell incubator (37°C).
8. After 30' add additional media (100µL per well, with or without test compounds). Remove bubbles.
9. Place assay plate into the IncuCyte ZOOM® and allow it to equilibrate for 5 min. Schedule repeat scanning (e.g. every 2-3h for 48h) in the ZOOM software: ensure 'scan type' is set to **Scratch Wound** and **Wide Mode** is selected if using 20x or 10x objectives.

Example invasion protocols (Matrigel, collagen-1), with full details of each of these steps for a representative cell type are described, in Section 5.

96-Well Scratch Wound Cell Migration/Invasion Assays: Schematic Diagram

Migration assays can be performed either with or without a baselayer extracellular matrix (**ECM: Step 1**), and do not utilize a gelled top layer (**eliminate step 4**). Test samples are added in culture media (**Step 5**). Protocols for using either

collagen-1 or Matrigel as the base/top layer are provided. In each cases test samples are made up in the relevant ECM (**Step 4**), as well as the additional culture media (**Step 5**).

1



Coat plate with with collagen-1 (or other ECM) and incubate overnight.

2

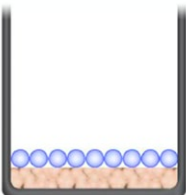
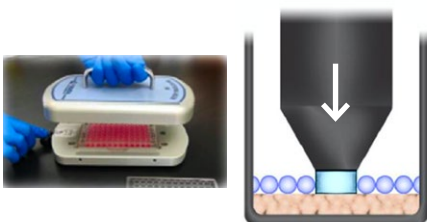


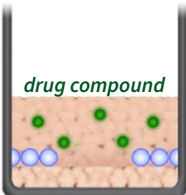
Plate cells on top of thin-layer matrix, and allow them to adhere for several hours.

3



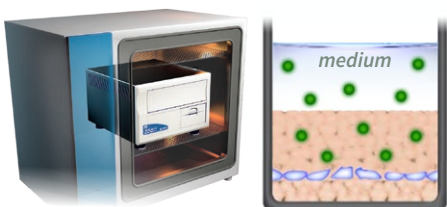
Create wound area using Essen® 96-well WoundMaker™ in just seconds.

4



Layer collagen gel on top of cells to create 3D matrix.

5



Use the Essen IncuCyte ZOOM® to:

- Automatically collect time-lapse images.
- Observe cell morphology.
- Quantify cell invasion.

96-Well Scratch Wound Cell Migration & Invasion Assays: Data Acquisition & Analysis

The IncuCyte ZOOM® 96-Well Scratch Wound Cell Migration and Invasion data processing software module allows automated detection and quantification of wound properties

from each well of the 96-well plate, at a series of time points post wounding. Kinetic plots of cell migration (movement into the wounded zone) can thus be readily generated.

To initiate and schedule the frequency of the image acquisition, the user must:

- 1) Prepare the assay plate as described in the relevant migration or invasion assay protocol.
- 2) Position the plate in the IncuCyte ZOOM®, close the drawer and the Incubator door.
- 3) Under 'Schedule Scans' select the tray position of interest. Remove any old tray definitions by clicking 'Remove Tray'. Click the 'Add Vessel' button and select '**96-well Essen ImageLock™**' microplate and the tray location of choice (left side or right side).
- 4) From the 'Scan Type' dropdown select '**Scratch Wound**'
- 5) If you would like to acquire fluorescence images (green and/or red) in addition to phase contrast images select the appropriate 'Channel Selection' check boxes.
- 6) Select the desired scan frequency and timing by, right clicking on the time base and selecting '**Set Interval**'.

For both Cell Migration and Cell Invasion applications, the first scan in the time course is used to generate the **initial scratch wound mask** (a digital overlay showing the border/leading edge of the migrating cells and the non-wounded area). This initial scratch wound mask is used in subsequent quantification processes and is critical to the success of the assay. Scratch wound masks are also computed for all subsequent image time points after the first scan.

In addition to the scratch wound mask, a **confluence mask** is also generated. The confluence mask represents the cell confluence of the wound region exactly as determined by the confluence algorithm. Each of these masks can be viewed either alone or in a blended combination within the 'Vessel View' window inside the IncuCyte™ software.

For full details of the IncuCyte ZOOM® 96-Well Scratch Wound Cell Migration and Invasion data processing please refer to the **IncuCyte ZOOM® Scratch Wound Processing Overview Technical Note**.

A summary of the data analysis process is given below.

- (1) Create an Image Collection of 3 to 6 representative images selected from the data set
- (2) Use the Image Collection to define and optimise a Processing Definition
- (3) Apply the Processing Definition to the full data set by running an Analysis Job

96-Well Scratch Wound Cell Migration Assays

An Image Collection is a group of images that can be used to train, test, or refine a Processing Definition. Image collections should contain approximately 3 to 6 images that represent the phenotypes under investigation for the selected time range. It is recommended that each test condition is represented in the image collection for Invasion or Migration studies. Depending on the experimental design, this may include multiple cell types, biomatrix materials, and both migration and invasion phenotypes. It is acceptable to include up to 10 images within an image collection. However, for larger image collections it will take longer to preview changes when optimizing a Processing Definition. If an experiment is performed using an objective of different magnification then a new image collection and processing definition will need to be made in order to analyse the resulting images.

The Processing Definition

The Scratch Wound Processing Definition uses an algorithm to generate two masks for each image of the image collection 1) the **scratch wound mask** and 2) the **confluence mask**. Scratch wound and confluence masks can be adjusted by altering the processing definition parameters. Changes to a single parameter (e.g. the position of the segmentation adjustment slider) can affect both the scratch wound and the confluence masks for an image. Guidance for optimizing Scratch Wound Processing Definitions can be found in the **IncuCyte ZOOM® Scratch Wound Processing Overview Technical Note**. After saving the Processing definition, it can be used to launch an Analysis Job.

The Analysis Job

When a processing job has been initiated for either a Cell Migration or Cell Invasion experiment, each image from the selected time range is analysed in accordance with the parameters chosen for the processing definition.

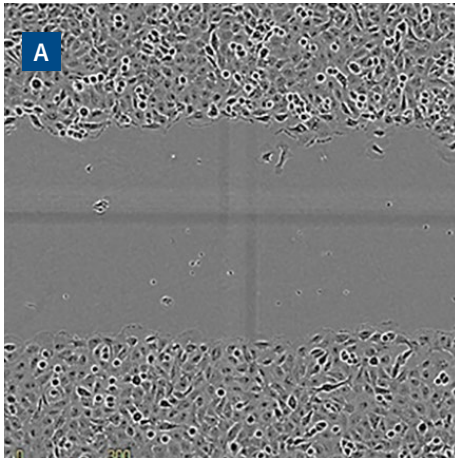
The first image from each well for the analysed time range is used to generate an **initial scratch wound mask** which defines the initial wound region. The initial scratch wound mask can be visualised as a digital overlay showing the cell free (wound) area at t=0h and is used to define the starting locations of the wound borders. The initial scratch wound mask is used in subsequent quantification processes and is critical to the success of the assay if relative wound density or wound confluence are used as metrics.

Further **scratch wound masks** are computed for each subsequent image in the series and are used to calculating the wound width metric only.

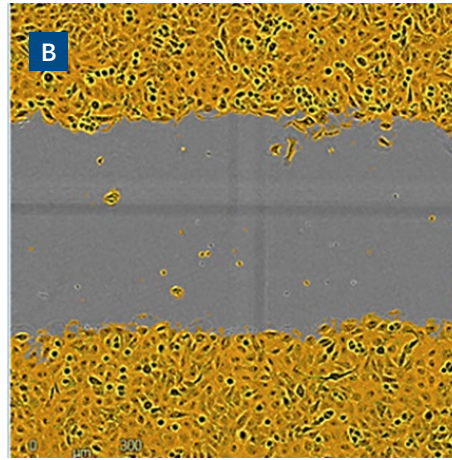
The **confluence mask** is used to derive the wound confluence metric (the cell confluence of the wound region). The wound region is defined by the initial scratch wound mask. Each of these masks can be viewed either alone or in a blended combination when an Analysis job is opened.

Wound Mask Images Using a 10x Objective

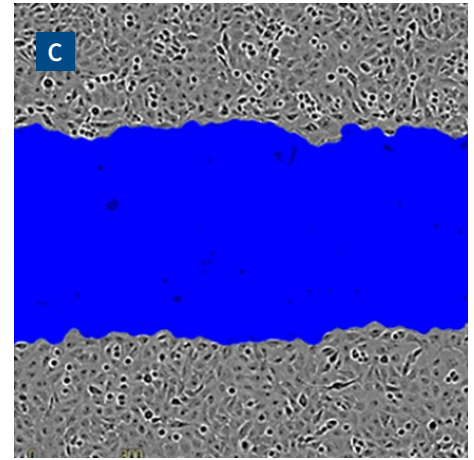
Initial wound (t=0h)



A Phase contrast images of HT1080 cells taken at 0h post wounding.

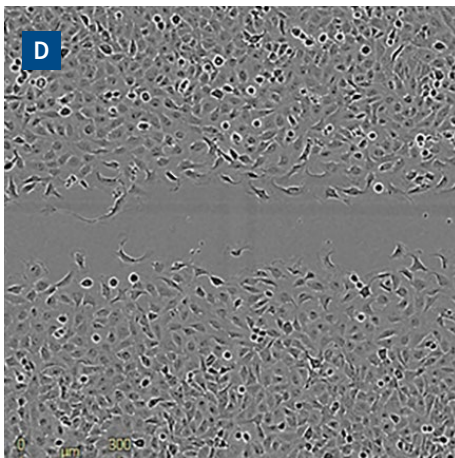


B Corresponding confluence masks (orange overlay blended with phase image) taken at 0h post wounding – indicate areas of the image that are occupied by cells.

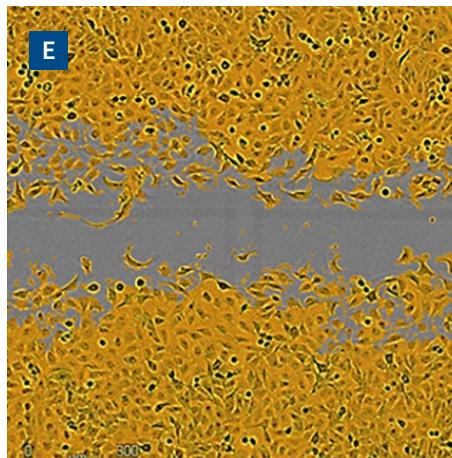


C Initial scratch wound mask (blue) superimposed on the phase image.

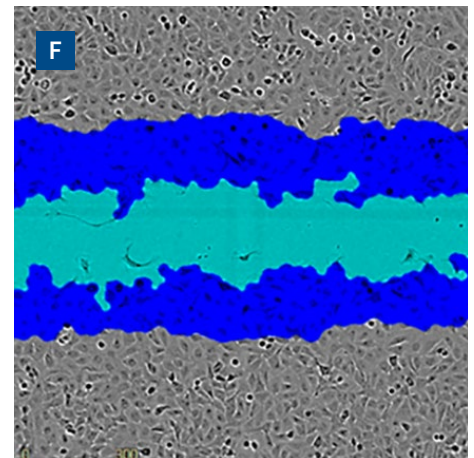
After 4h



D Phase contrast images of HT1080 cells taken at 4h post wounding.



E Corresponding confluence masks (orange overlay blended with phase image) taken at 4h post wounding – indicate areas of the image that are occupied by cells.



F Scratch wound mask (light blue) indicating the wound border locations after 4h is overlaid on the initial scratch wound mask (blue).

Initial Scratch Wound Mask: Identifies the borders of the wound region using the first image in a temporal series.

Scratch Wound Mask: Identifies the leading edge of the population of migrating cells within each image of the series.

Confluence Mask: Identifies all cell occupied areas within the field of view and is used in combination with the Initial Scratch Wound Mask to determine the cell confluence of the wound region.

IncuCyte™ Software: Scratch Wound Cell Migration & Invasion Analysis Metrics

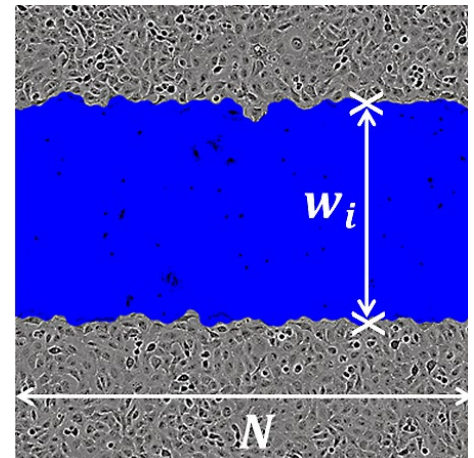
There are three integrated metrics that the software calculates based on the processed images:

1. Wound Width (cell migration only)

Wound Width represents the average distance (microns) between the edges of the scratch wound mask in each line of resolution within an image. This is the only metric of the three available metrics that does not rely on the initial scratch wound mask, as each image is analyzed independently of the starting point. It is important to note that the scratch wound mask identifies the boundary of the migrating cell population and, at times, will ignore a small number of cells that do not fit within that population.

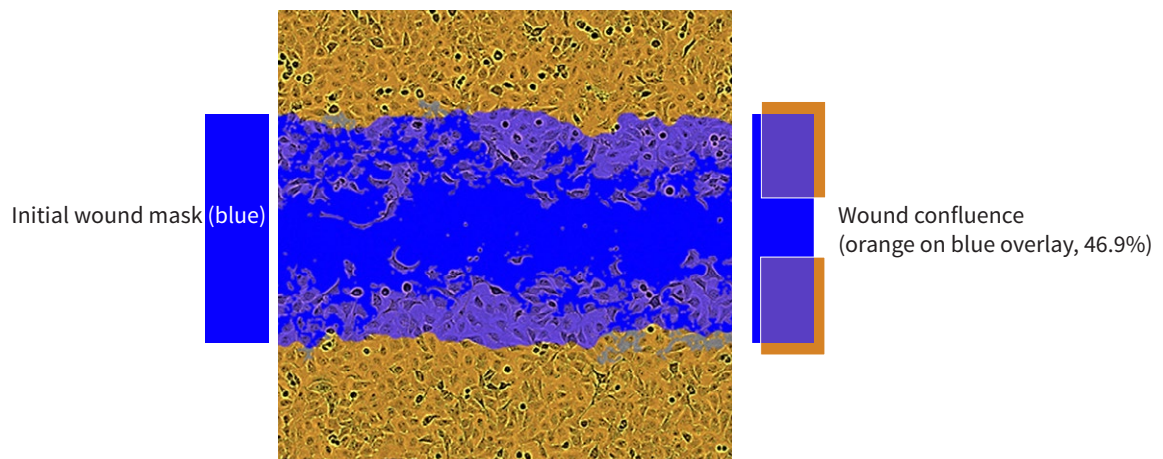
$$\text{wound width} = \frac{1}{N} \sum w_i$$

Wound width (µm) is determined using each vertical line of resolution of a scratch wound mask.



2. Wound Confluence (cell migration only)

Wound Confluence measurement relies on the initial scratch wound mask to differentiate the wounded from the non-wounded region. The Wound Confluence algorithm determines the cell confluence in only the wound region. The resulting value represents the percentage of wound area that is occupied by cells. Cells, debris or other matter within the wound is quantified as confluence and is not background subtracted. Therefore, the wound confluence values at the initial time point may not be 0% and may vary from well-to-well.



Wound confluence (%) represents the fractional area of the wound that is occupied by cells.

3. Relative Wound Density (cell migration and cell invasion)

Like Wound Confluence, **Relative Wound Density (RWD)** also relies on the initial scratch wound mask to differentiate between cell-occupied and cell-free regions of the image. Once these regions are defined, a second image analysis algorithm (which is completely independent of the confluence algorithm and is not viewable to the user) is applied to calculate the density of both the cell region and the wound region as defined by the initial scratch wound mask. It is important to note that both the cell region and the wound region are variables in the Relative Wound Density v1.0 equation, and both are treated with equal importance. Specifically, the RWD metric refers to the density of the wound region as a function of the density of the cell region and is defined by the following equation:

$$\%RWD(t) = 100 \cdot \frac{(w(t) - w(0))}{(c(t) - w(0))}$$

$w(t)$ = Density of wound region at time, (t)

$c(t)$ = Density of cell region at time, (t)

Note that that the initial density of the wound ($w(0)$) is subtracted from the density of the wound at each subsequent time point ($w(t)$) i.e. each reported value is background subtracted. The background subtracted wound density represents the numerator in the above equation. For the most part, the density of the cell region ($c(t)$) represents the denominator in the above equation as the initial density of the wound ($w(0)$) is typically very small. The reason $w(0)$ is subtracted from $c(t)$ is to guarantee that the relative wound density value is equal to 100% when $w(t)$ is equal to $c(t)$.

Relative Wound Density accounts for the background density of the wound at the initial time point, and for changes in both the density of the cell (non wound) and the wound region. Thus, at the initial time point RWD is always 0%. RWD is particularly useful when experimental treatments alter cell morphology. In as much as RWD does not rely on the identification of individual cell borders it is also helpful when using cell types that are difficult for the Wound Width and Wound Confluence algorithm to accurately evaluate (e.g. cells with flattened low contrast morphology for example certain human umbilical vein endothelial cells).

Relative Wound Density is a measure (%) of the density of the wound region relative to the density of the cell region.

Why is RWD the only recommended metric for Cell Invasion?

There are several differences between cell migration and cell invasion, both in experimental design and biological phenotypes. There are two reasons that only RWD is suitable for cell invasion:

- 1) Invasion assays require the addition of an extracellular matrix (ECM) for cells to invade. The presence of an ECM can hinder the ability of the analysis algorithm to apply an appropriate initial scratch wound mask and confluence mask to the first image of a series. Cellular content or textured regions of ECM within the wound area can result in a misleading wound confluence metric. In addition, the mild texture of gelled ECM can cause intermittent over- and underestimations of confluence in the wound area resulting in variable data. The RWD algorithm is more tolerant of ECM texture and will correct for initial cell content in the wound area.
- 2) Cells invading through a 3D matrix exhibit very different morphology from cells migrating over a 2D substrate. Cells with a mesenchymal invasion morphology typically display an elongated phenotype with lamellipodia that extend into the ECM. Furthermore, one leader cell is generally followed by numerous other cells, thereby forming a network of invasion tracts or 'tunnels', as opposed to a leading edge of cells seen in a 2D migration assay. For this reason, the scratch wound masks may not always best represent the invading population: wound width measures can thus be inaccurate and misleading.

Hints and tips

Optimizing Assays

For optimal assay quality, for both IncuCyte™ 96-Well Scratch Wound Cell Migration and Cell Invasion assays it is recommended that cell density, the timing of the scratchwound (post cell plating) and the density of biomatrix material (if required) be investigated and optimized for different cell types.

1. Cell density

- The most consistent wounds are generally made when the cell monolayer is at or very near to 100% confluence, with nearly all cells attached to the tissue culture plate just prior to wounding.
- It is best if the cells are not at confluence for an extended period of time prior to wounding.
- We recommend using the minimum number of cells that are required to form a confluent monolayer within 6 – 18h of plating.
- Seeding density will vary with each cell type used, i.e. if the cells are large, a smaller number will be required to achieve confluence. Typically this will range from 10-50K cells per well.
- Proliferation rates can also affect seeding density. It is advisable that you complete a measure of proliferation using the confluence metric within the IncuCyte™ to assist in defining an appropriate seeding density.

2. Timing of the Scratchwound

- The optimal timing depends on many factors including the speed at which the cells adhere to the plastic (or biomatrix), the strength of this adhesion, the number of cells plated, and the proliferation rate of the cells to name just a few.
- Plating cells at the end of the day and wounding cells in the morning of the following day works well for many cell types plated on tissue culture plastic. This is a good place to start once an optimum seeding density has been identified.
- If a biomatrix material is being utilized, cells may adhere in just a few hours, and successful wounding is often possible on the same day of seeding.

3. Biomatrix coating

- Coating the well with a biomatrix material (e.g. Collagen-I) or poly-D-Lysine will typically enhance the speed and strength of cell attachment. It can allow cells to adhere more tightly to the substrate as opposed to each other which can facilitate the removal of only cells in the wound area avoiding cell sloughing or the removal of sheets of cells.
- On occasion, cells will adhere too tightly. Sub-optimal wounding may then occur when cells leave adhered debris after wounding, which blocks subsequent cell migration. **Plating cells for shorter time periods, to allow only partial attachment (e.g. 4 – 8h)** can help improve the quality of the wounds.

For a detailed description of an example optimization strategy, please refer to the **Scratch Wound™ 96-Well Cell Migration and Invasion Assays Application Note**.

Working with Collagen-1 and Matrigel

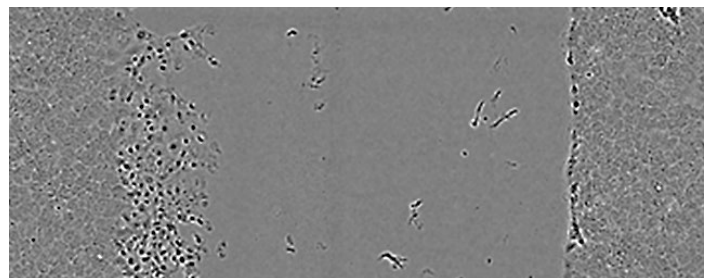
Working with either Collagen-1 or Matrigel can be challenging, given their pH/temperature dependent viscosity and known batch-to-batch variation. To maximize performance and cost effectiveness, we recommend:

- Always keep working stocks on ice, or when in plates, in the CoolSink.
- Use wide bore pipette tips (cut off) and serological pipettes as much as possible – the high viscosity of collagen-1/Matrigel makes them hard to pipette through narrow tips (e.g. 100µL). Store tips in the fridge – this will help keep everything cool.
- Plan for larger dead volumes than usual.
- Neutralizing collagen-1 with neutralization buffer takes time, and very careful and complete mixing. Try to minimize the number of neutralization manipulations – e.g. neutralize once in a larger volume and then dispense to multiple assay wells to create on-plate replicates, rather than neutralizing as individual wells. This also helps to minimize wastage from dead volume
- Once neutralized, collagen-1 will slowly gel even when kept cold (within 30'). Working quickly and efficiently will help ensure good results.
- When preparing plates containing test samples, it is more straightforward to make serial dilutions in solvent (e.g. DMSO) which are then transferred to a 96-well microplate (e.g. 1µL per well) and diluted in ECM/neutralization buffer/culture media as appropriate. Making serial dilutions in ECM is time consuming and difficult.
- Due to the high freezing point of DMSO, addition to cold Matrigel creates the potential for samples to freeze, and then precipitate. In our experience keeping DMSO: Matrigel ratios of <1:100 (i.e <1%) mitigates this possibility.
- Different batches (lots, suppliers) of collagen-1 and Matrigel may yield different experimental outcomes. We recommend that batches <3 months old are used to minimize variability. Both Standard (BD 354234) and Growth Factor Reduced (BD 354230) Matrigel have been validated for use.

Troubleshooting

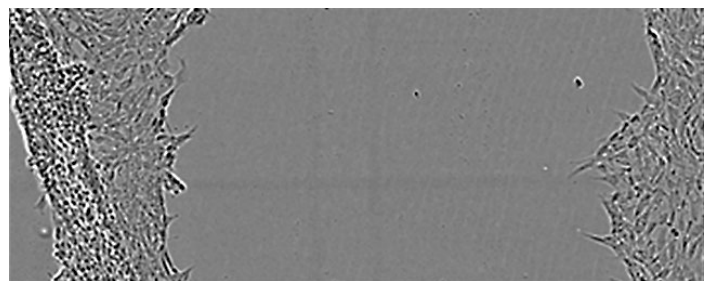
1. Cells are not completely removed by the WoundMaker™

Some cells may adhere strongly to the plastic/culture plate surface. Try minimizing the plating time (i.e. reduce to 4-6h) to reduce strong adhesion.



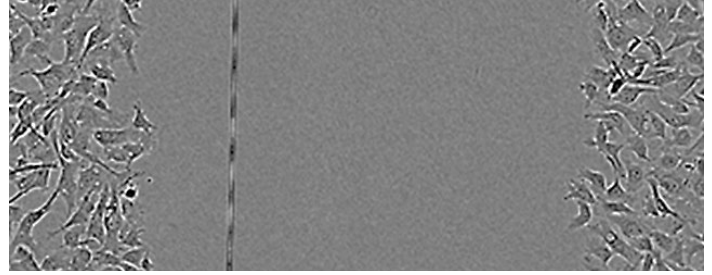
2. Cells are removed in large sheets during the scratch wound assay

Loosely adherent cell types may be removed in sheets by the WoundMaker™ or slough off during washes. Some cell types adhere tightly to neighboring cells but only loosely to the substrate. Try coating the plate with Matrigel or collagen-1 prior to seeding cells to promote stronger adherence. If cells adhere tightly to one another, try allowing the cells to adhere for a shorter period of time (four to six hours) before wounding.



3. Pin scratches into the culture surface along wound

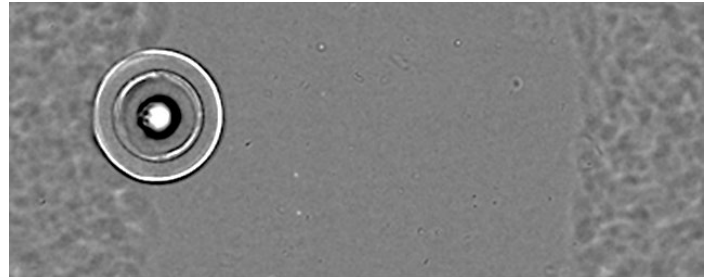
Even under ideal conditions, dust and particulate matter (grit) may come into contact with and become imbedded in the soft PTFE tips of the WoundMaker™'s pins. The standard washing protocol is often sufficient to remove grit. If a pin consistently scratches into the culture surface, pin cleaning or replacement at Essen BioScience may be required.



4. Bubbles form within gel layer during an invasion assay

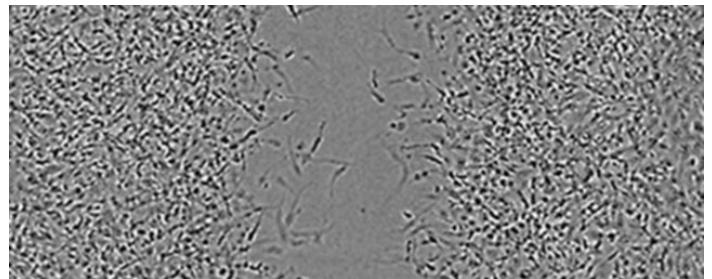
Due to its viscosity, the gel layer of a matrix material may retain bubbles that form during pipetting but would normally escape to the surface. When using collagen-1, bubbles may also form during neutralization due to pipetting and the formation of CO₂. Take extra care when pipetting into matrix materials, especially at high concentrations.

For collagen-1, centrifugation of the neutralized solution and careful pipetting will help eliminate bubbles. Bubbles can also be removed from wells by gently squeezing a wash bottle (containing 100% ethanol with the inner straw removed) to blow vapor over the surface of each well.



5. Cells appear out of focus during an invasion assay

Cells that are plated too densely can lead to the deformation of the biomatrix material causing cells adhered to it to become out of focus. This can lead to difficulties in image analysis. Careful optimization of cell density can resolve this problem. It is advisable to use the minimum number of cells required to achieve 80-100% confluence at the time of wounding.



6. No wound is formed in the cell layer

Occasionally, a pin will fail to fully contact the cell surface and create a wound. This is usually due to build-up of precipitate along the length of the pin, preventing the pin from moving freely. Wash the WoundMaker™ thoroughly. If a pin consistently fails to create a wound, the WoundMaker™ will need to be serviced at Essen BioScience.

7. Cells migrate in presence of gel layer

Cell migration in the presence of a gel layer, when invasion should be observed, is indicative of improper gelling of the matrix material or a lack of bonding to the thin layer. Ensure ideal experimental conditions. While working with matrix materials, keep them strictly under conditions that do not promote gelling. Use a CoolSink 96F during gelling to apply an even temperature across the bottom of the plate.

Detailed Scratch Wound Cell Migration Protocol for HT1080 cells

Detailed Demonstration Protocol

This protocol describes the measurement of HT1080 osteosarcoma migration in a single 96-well plate, without test samples.

Materials

- Growing stocks (T75, 37°C, 5% CO₂) of HT1080 Osteosarcoma cells (ATCC: CCL-121) in:
- 100ml of DMEM cell culture media + 10% FBS + 0.1mM NEAA + 1mM sodium pyruvate
 - DMEM (Life Technologies 32430)
 - FBS Fetal bovine serum, characterized (Thermo Scientific - HyClone SH3007103)
 - 10mM NEAA (Life Technologies 11140)
 - 100mM Sodium pyruvate (Life Technologies 11360)
- 96-well ImageLock™ plate (Essen BioScience: 4379) 1 per assay
- 96-well microplate (Corning: 3595) 1 per assay
- Trypsin/EDTA (Life Technologies 25200)
- D-PBS (w/o Ca²⁺, Mg²⁺, pH 7.0 - 7.3)

Protocol

Day 1 – Seed cells (30 min, wait 18h)

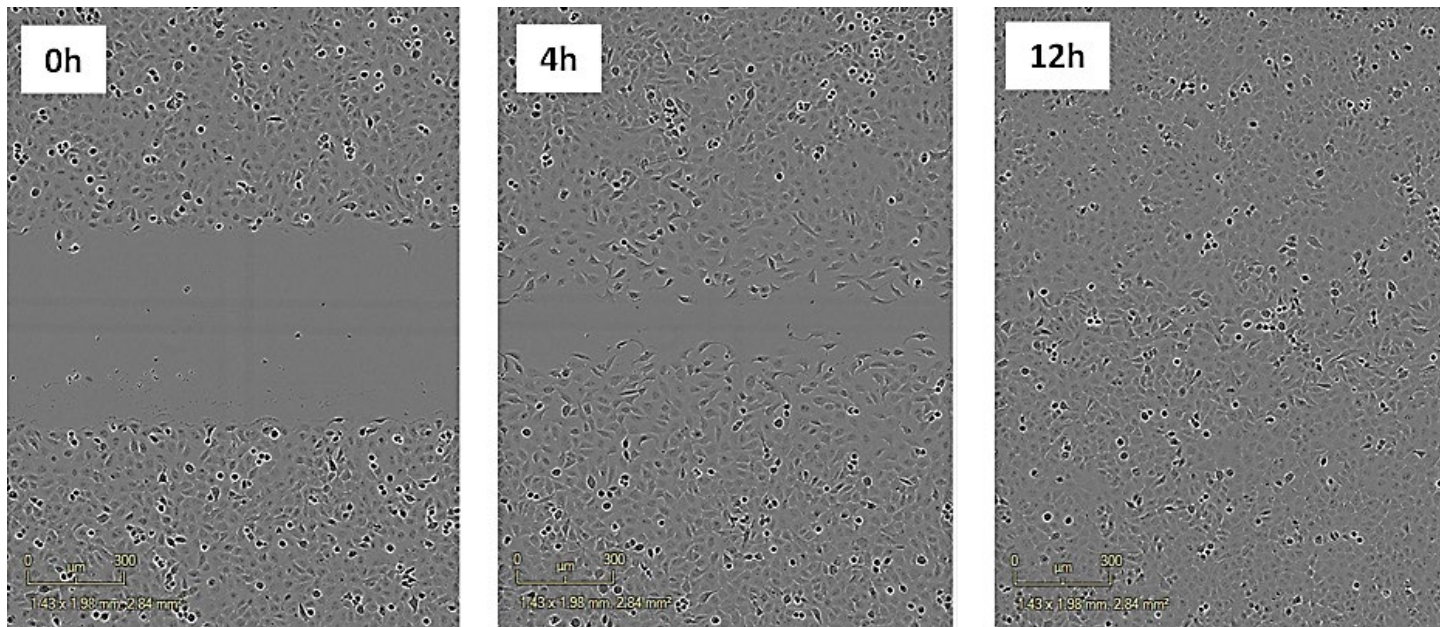
1. Remove a freshly confluent T75 flask of HT1080 cells from the cell incubator and place within a sterile cell culture hood.
2. Remove media, and wash cells with D-PBS (10ml).
3. Add 1ml Trypsin/EDTA to detach cells, leave for 2-3min.
4. Add 9ml of culture media, and re-suspend cells using a 10ml serological pipette.
5. Perform a cell count (e.g. trypan blue staining + haemocytometer). Centrifuge the cell suspension (185 ×g, 4 min) and re-suspend the cell pellet in culture media at 2.5x10⁵ cells ml⁻¹.
6. Plate cells (100 µL per well, i.e. 25K cells) into every well of an **ImageLock™ plate**. Do not leave any empty (dry) wells – these will damage the WoundMaker™. Allow the cell plate to sit at room temperature for 5min to allow cells to evenly disperse across the bottom of the plate. Place the lidded plated into a 37 °C incubator, 5% CO₂ for **18h**.

DAY 2 – Wound, add samples & schedule scans (20 – 30min)

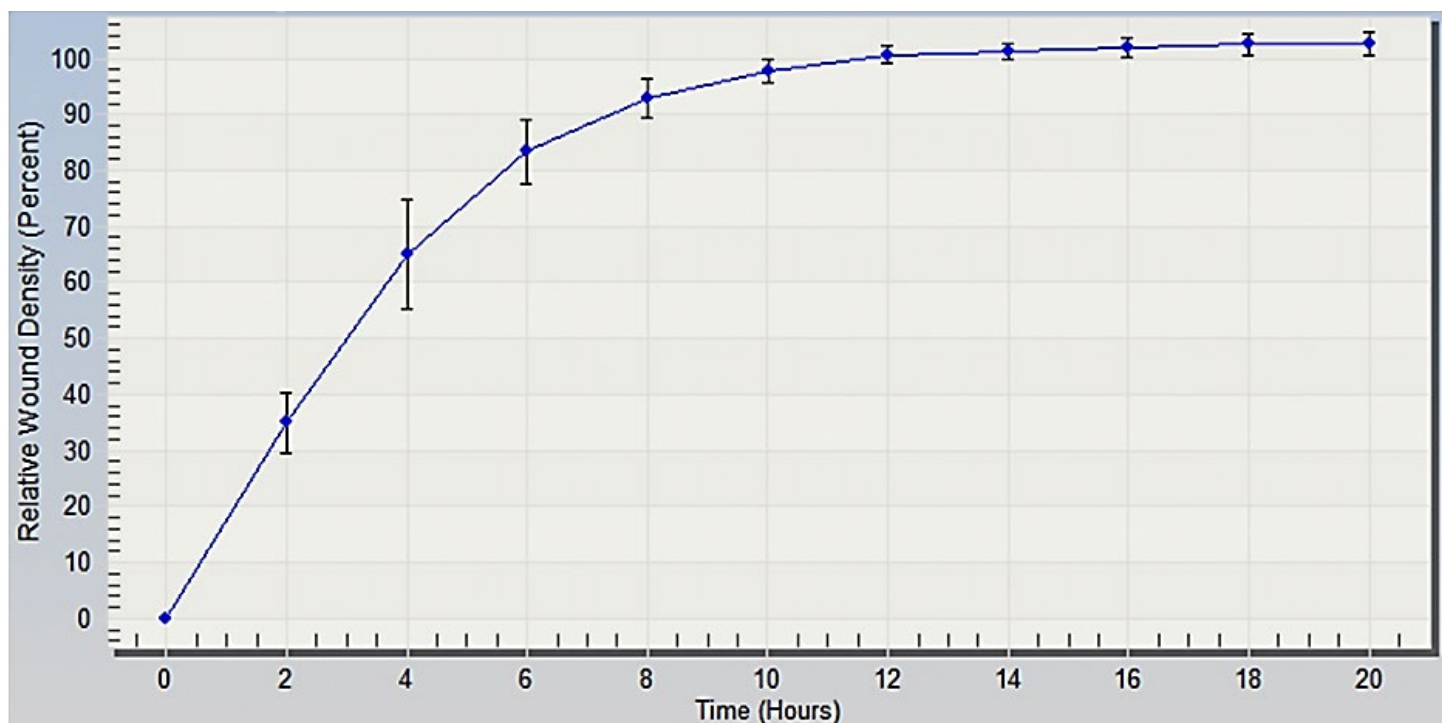
1. Carefully remove the ImageLock™ plate from the incubator.
2. Follow the 96-well WoundMaker™ procedure to simultaneously create wounds in all wells. Visually inspect wounds with an inverted microscope, to ensure that you have correctly used the WoundMaker™.
3. Immediately (and carefully) wash the cells twice with culture media (100 µl per well).
4. Using a multi-channel pipette, add 100 µl of culture media (or test sample from the COMPOUND PLATE) to each well.
5. Remove bubbles from all wells by gently squeezing a wash bottle (containing 100% ethanol with the inner straw removed) to blow vapor over the surface of each well. Try to keep the tip of the wash bottle approximately 5cm from the media surface.
6. Place the de-bubbled cell plate in the IncuCyte ZOOM®, close the drawer and incubator door.
7. Allow the cell plate to equilibrate in the IncuCyte ZOOM® for 10min prior to scheduling the first scan – this will reduce the likelihood of condensation on the lower side of the plate which can hinder image quality.
8. In the IncuCyte ZOOM® software, schedule repeat scanning for every 2h for 24h, with the first scan to commence immediately. Set the assay plate to scan one image per well (Wide Mode if available) and ensure ‘scan type’ is set to Scratch Wound.
9. Wash and store the WoundMaker™ according to the established Wash Protocols.

Detailed Scratch Wound Cell Migration Protocol: HT1080 cells

Illustrative data: HT1080 96-Well Scratch Wound Cell Migration



Representative phase contrast images of HT1080 cells taken at 0h, 4h and 12h post wounding. Note initial confluence, clean wound boundaries and symmetrical migration pattern.



Summary data time course of migration (% relative wound density; points are mean values and vertical bars show standard deviation).

Detailed Scratch Wound Cell Invasion Protocol for Matrigel + HT1080 cells

Detailed Demonstration Protocol

This protocol describes the measurement of HT1080 osteosarcoma cell invasion and (control) migration in 1 x 96 well plate, without test samples. 50% of wells are used to measure invasion and 50% migration.

Materials

- Growing stocks (T75, 37°C, 5%CO₂) of HT1080 Osteosarcoma cells (ATCC: CCL-121) in:
- 100ml of DMEM cell culture media + 10% FBS + 0.1mM NEAA + 1mM sodium pyruvate
 - DMEM (Life Technologies 32430)
 - FBS Fetal bovine serum, characterized (Thermo Scientific - HyClone SH3007103)
 - 10mM NEAA (Life Technologies 11140)
 - 100mM Sodium pyruvate (Life Technologies 11360)
- 96-well ImageLock™ plate (EsSEN BioScience: 4379) 1 per assay
- 96-well microplate (Corning: 3595) 1 per assay
- Trypsin/EDTA (Life Technologies 25200)
- D-PBS (w/o Ca²⁺, Mg²⁺, pH 7.0 - 7.3)
- Matrigel standard (BD 354234)
- IncuCyte™ 96-Well Scratch Wound Cell Invasion Accessories (EsSEN: 4444) include:
 - 2 x CoolBox 96F System plus 2 x CoolSink 96F (EsSEN: 1500-0078-A00)
 - 1 x extra CoolSink 96F (EsSEN: 1500-0080-A00)
 - CoolBox M30 System with CoolRack (EsSEN: 1500-0079-A00)

Protocol

IMPORTANT:

In advance of the experiment it is important to have stored the Cool pack accessories at the correct temperatures for at least 4h:

Coolbox x2 (block with gelpack: -20°C), Coolsink 96F x2 (4°C), Coolsink 96F x1 (37°C).

CoolBox M30 System (block with gelpack: -20 °C) with CoolRack (4°C).

The Cool Packs are used to ensure close temperature control of Matrigel in microplate plates. Matrigel stock (standard or GFR) is stored at -20°C. At 4 - 8 °C, Matrigel is a viscous liquid. Gelling will occur slowly at 4 - 8°C, and more rapidly when warmed to 37 °C. For this reason it is imperative to store Matrigel working solutions at 4-8 °C at all times to avoid unwanted gelling. It is easier to handle low volume (<500µL) Matrigel solutions using pre-cooled (from a fridge), wide bore pipette tips or serological pipettes. Ahead of the experiment, Matrigel (standard and GFR) can be defrosted and stored at -20°C in 1ml aliquots.

Matrigel batches are supplied at a range of concentrations. We recommend sourcing a batch of Matrigel with a concentration of >9mg/ml and an endotoxin level of <3 (EU)/ml. The following protocol assumes a Matrigel stock concentration of 9.6 mg ml⁻¹.

Day 1 – Thaw Matrigel

1. Defrost Matrigel by placing the bottle into an ice bucket which is then placed in the fridge (+ 4°C) overnight. When fully thawed there should be no gel clumps visible. If aggregates are present replace the bottle on ice and thaw at + 4°C for a longer period of time. After thawing, chill ten 1ml eppendorf tubes in the CoolSink M30 System (10min). Using a pre-cooled serological pipette (10ml) aliquot (1ml) the Matrigel into each tube and store at -20°C.

Day 2 – Coat the ASSAY PLATE with thin layer of Matrigel (10min, wait 1d)

1. Using the CoolSink M30 System with CoolRack, thaw one 1ml aliquot of 9.6 mg ml⁻¹ Matrigel (~1 h). In a 15 ml Falcon tube, dilute Matrigel stock to 100 µg ml⁻¹ in HT1080 culture media by slowly pipetting 63 µl of 9.6 mg ml⁻¹ Matrigel into 5.937 ml culture media. Pipette five times to mix, ensuring any residual Matrigel in the pipette tip has been washed into the vial.
2. Dispense 5 µl of diluted Matrigel into each well of a 96-well ImageLock™ plate. Gently rock the plate to ensure even coating of each well. Place the plate in a 37 °C incubator, 5% CO₂ overnight.

Detailed Scratch Wound Cell Invasion Protocol: Matrigel + HT1080 cells

Day 3 – Seed cells onto the ASSAY PLATE (30min, wait 4h)

1. Remove a freshly confluent T75 flask of HT1080 cells from the cell incubator and place within a sterile cell culture hood.
2. Remove media, and wash cells with D-PBS (10ml).
3. Add 1ml Trypsin/EDTA to detach cells, leave for 2-3min.
4. Add 9ml of culture media, and re-suspend cells using a 10ml stripette.
5. Perform a cell count (e.g. trypan blue staining + haemocytometer).
6. Centrifuge the cell suspension ($185 \times g$, 4 min) and re-suspend the cell pellet in culture media at 2.5×10^5 cells ml⁻¹.
7. Remove ASSAY PLATE from the incubator and aspirate and discard the Matrigel from each well and seed cells (100 μ l per well, i.e. 25K cells per well) into every well. Do not leave any empty (dry) wells - these will damage the WoundMaker™.
8. Allow the cell plate to sit at room temperature for 5min to allow cells to evenly disperse across the bottom of the plate. Place the lidded plate into a 37 °C incubator, 5% CO₂ **for 4h**.

Day 3 – Prepare the REAGENT plate (30min)

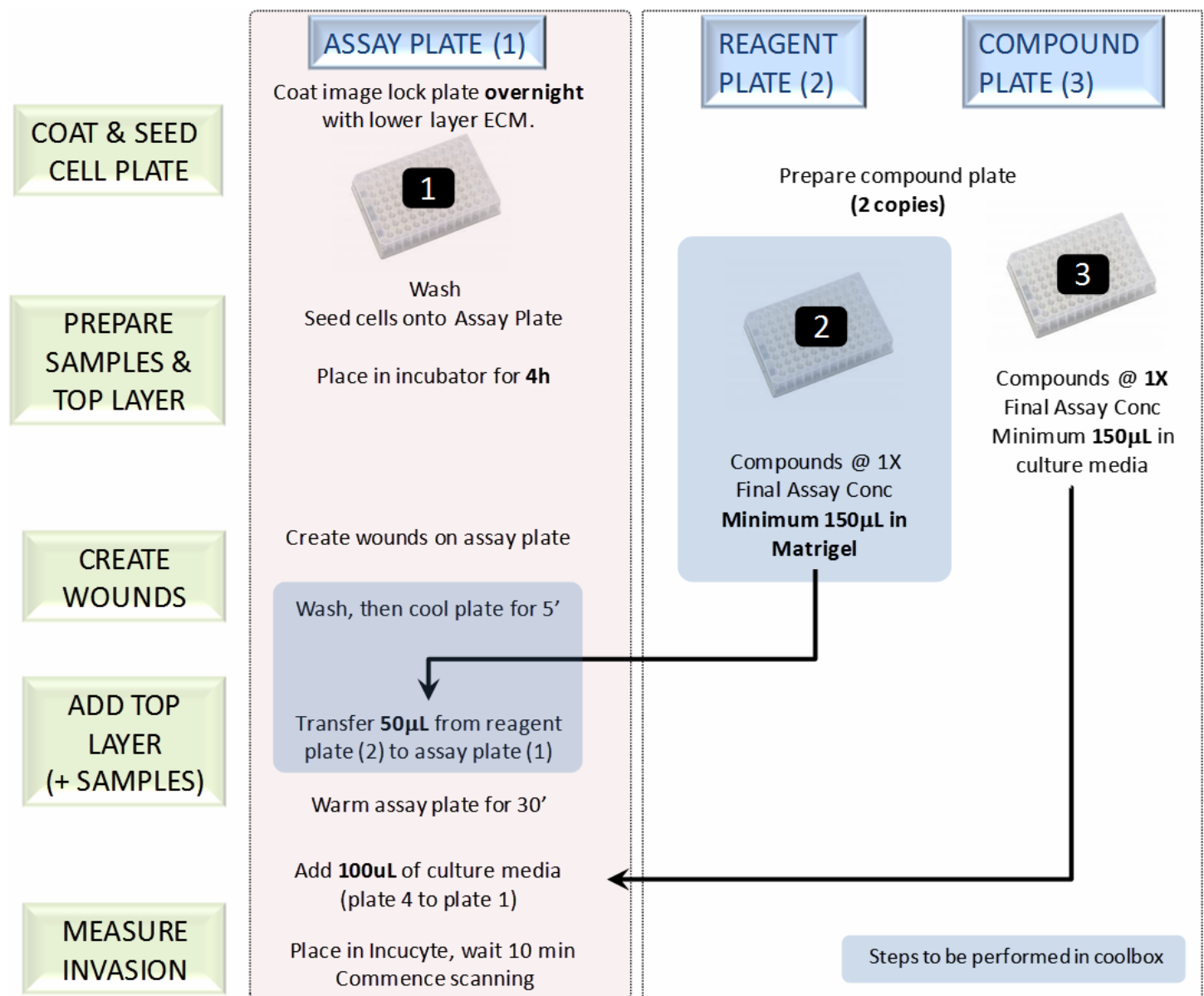
1. Place a standard 96-well plate in the pre-chilled CoolBox 96F at 4 - 8 °C for 5 min. Keep this plate on the CoolBox throughout the protocol.
2. Prepare a working stock (5ml) of 8 mg ml⁻¹ Matrigel in cold culture media. In a 15ml falcon tube add 4.17ml of 9.6 mg ml⁻¹ Matrigel to 0.83ml of cold culture media (+4°C). Pipette five times to mix, ensuring any residual Matrigel in the pipette tip has been washed into the vial. Keep on ice.
3. Add 200 μ L Matrigel working stock to each well of columns 1 & 4 (for Invasion wells).
4. Add 200 μ L of culture media to each well of columns 7 & 10 (for Migration control wells).
5. Remove bubbles from by gently squeezing a wash bottle (containing 100% ethanol with the inner straw removed) to blow vapor over the surface of each well. Try to keep the tip of the wash bottle approximately 5cm from the media surface.
6. **NOTE:** in sample testing experiments, the REAGENT PLATE should contain samples prepared (diluted) in Matrigel/ culture media, at the final assay concentration. A second plate containing compounds (the COMPOUND PLATE, prepared in media) also needs to be made.

Day 3 – Wound, add top layer & schedule scans (40 – 50min)

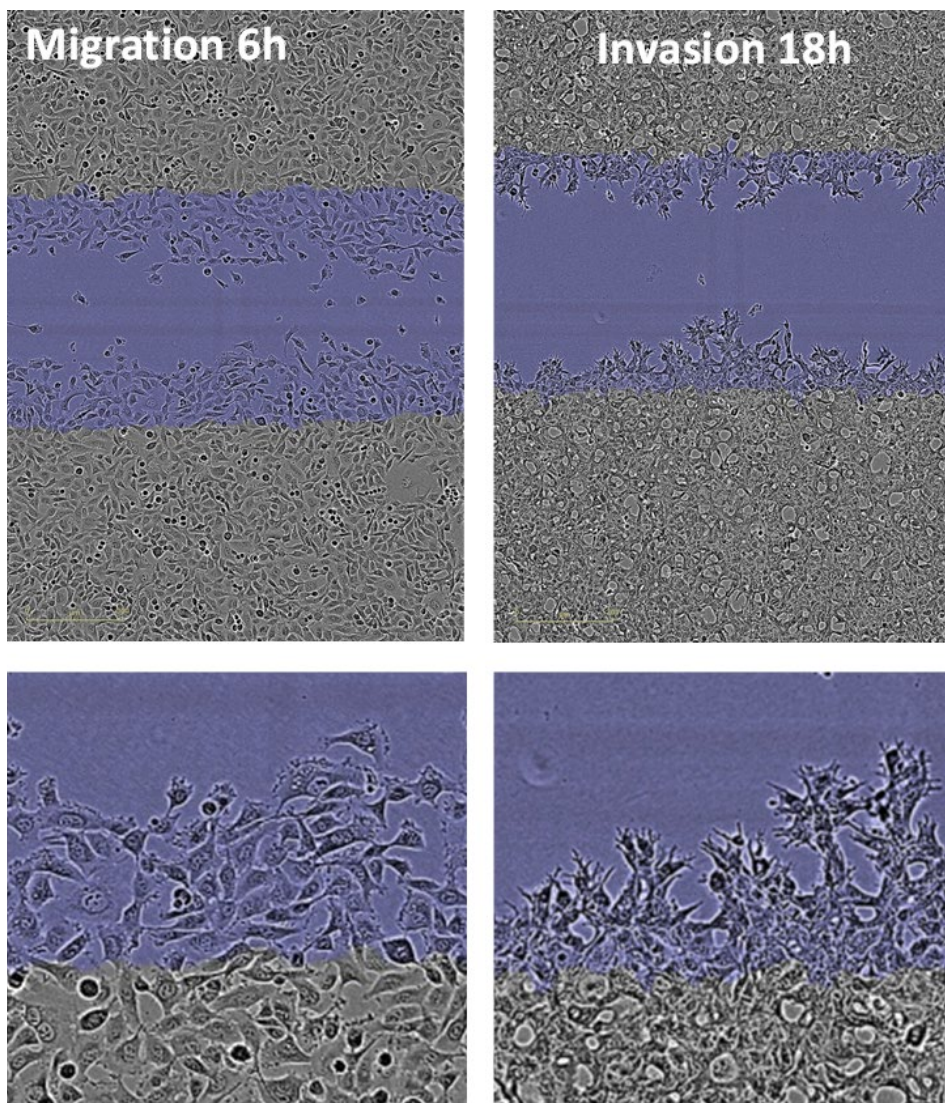
1. Carefully remove the ASSAY plate from the incubator.
2. Follow the 96-well WoundMaker™ procedure to simultaneously create wounds in all wells. Visually inspect wounds with an inverted microscope, to ensure that you have correctly used the WoundMaker™.
3. Immediately (and carefully) wash the cells twice with cell culture media. After the final wash, add culture media (100 μ l) to each well and place plate in a pre-chilled CoolBox 96F to equilibrate for 5 min. Remove and discard (cool) media from the ASSAY PLATE.
4. Transfer the Matrigel solution and culture media from the REAGENT PLATE to the ASSAY PLATE: 50 μ l per well Matrigel to Col 1 – 6 and 50 μ l per well culture media to Col 7 – 12. Remove bubbles as before.
5. Place the ASSAY PLATE on to the pre-warmed CoolSink 96F in a 37°C, 5% CO₂ incubator for 30 min.
6. Add 100 μ l of additional culture media to each well of the ASSAY plate. Do not mix. Remove bubbles. Place the de-bubbled cell plate in the IncuCyte ZOOM®, close the drawer and incubator door.
7. Allow the cell plate to equilibrate in the IncuCyte ZOOM® for 10min prior to scheduling the first scan – this will reduce the likelihood of condensation on the lower side of the plate which can hinder image quality.
8. In the IncuCyte ZOOM® software, schedule repeat scanning for every 3h for 48h, with the first scan to commence immediately. Set the assay plate to scan one image per well (Wide Mode if available) and ensure 'scan type' is set to Scratch Wound.
9. Wash and store the WoundMaker™ according to the established Wash Protocols.

Detailed Scratch Wound Cell Invasion Protocol: Matrigel + HT1080 cells

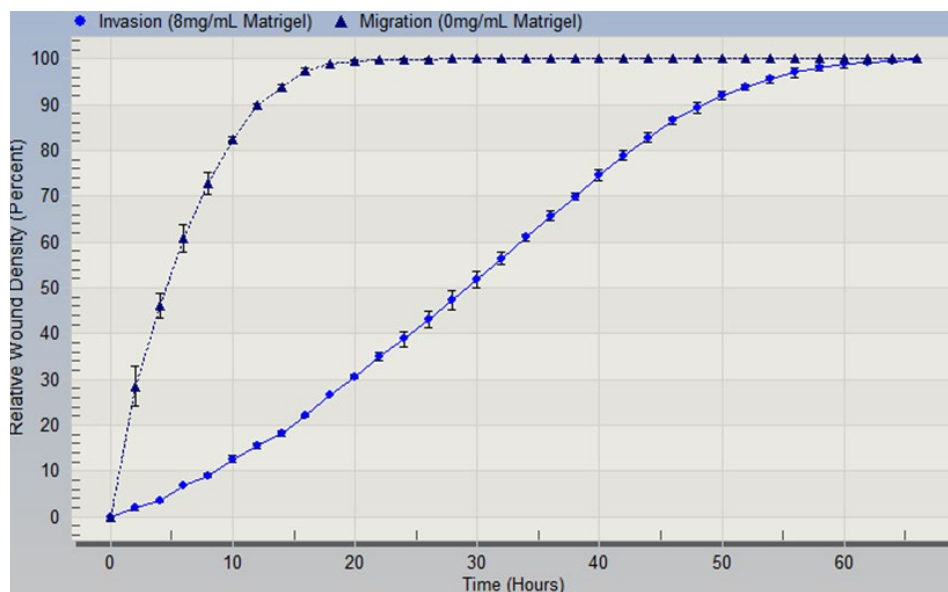
Schematic diagram of Matrigel Invasion Assay Workflow with test samples



Detailed Scratch Wound Cell Invasion Protocol: Matrigel + HT1080 cells



Representative phase contrast images of HT1080 cells (upper; 10X magnification, lower; digitally zoomed) taken at 6h post wounding for migration (left) and 18h post wounding for invasion (right). Note clean wound boundaries and symmetrical migration/invasion. Also note the characteristic differences in cell morphology.



Summary data time courses of migration and invasion (% relative wound density; points are mean values and vertical bars show standard deviation).

Detailed Scratch Wound Cell Invasion Protocol for Collagen-1 + HT1080 cells

Detailed Demonstration Protocol

This protocol describes the measurement of HT1080 osteosarcoma cells invasion and (control) migration in 1 x 96 well plate, without test samples. 50% of wells are used to measure invasion and 50% for control migration.

Materials

- Growing stocks (T75, 37°C, 5%CO₂) of HT1080 Osteosarcoma cells (ATCC: CCL-121) in:
- 100ml of DMEM cell culture media + 10% FBS + 0.1mM NEAA + 1mM sodium pyruvate
 - DMEM (Life Technologies 32430)
 - FBS Fetal bovine serum, characterized (Thermo Scientific - HyClone SH3007103)
 - 10mM NEAA (Life Technologies 11140)
 - 100mM Sodium pyruvate (Life Technologies 11360)
- 96-well ImageLock™ plate (EsSEN BioScience: 4379) 1 per assay
- 96-well microplate (Corning: 3595) 1 per assay
- Trypsin/EDTA (Life Technologies 25200)
- D-PBS (w/o Ca²⁺, Mg²⁺, pH 7.0 - 7.3)
- 7.5% solution of NaHCO₃ (Sigma S8761) and solid NaHCO₃ (Sigma S5761)
- Tissue culture grade (TC) distilled water
- 10X DMEM (Sigma D2429)
- 20 mM acetic acid
 - 230 µl glacial acetic acid (Sigma A9967) + 200 ml distilled water
- Collagen-1 (rat tail), 5 mg ml⁻¹ in 20 mM acetic acid (R&D Systems: 3440-100-0 or Trevigen: 3447-020-01)
- IncuCyte™ 96-Well Scratch Wound Cell Invasion Accessories (EsSEN: 4444) include:
 - 2 x CoolBox 96F System plus 2 x CoolSink 96F (EsSEN: 1500-0078-A00)
 - 1 x extra CoolSink 96F (EsSEN: 1500-0080-A00)

Protocol

IMPORTANT:

In advance of the experiment it is important to have stored the Cool pack accessories at the correct temperatures for at least 4h:

Coolbox x2 (block with gelpack:

-20°C), Coolsink 96F x2 (4°C), Coolsink 96F x1 (37°C).

CoolBox M30 System (block with gelpack:

-20°C) with CoolRack (4°C).

The Cool Packs are used to ensure close temperature control of collagen-1 in microplate plates. Collagen-1 is kept as a stock in dilute 20 mM acetic acid to maintain it in monomeric form. At 4 - 8°C, collagen-1 is a viscous liquid. To polymerize and form a gel, collagen must be neutralized to physiological pH. Following neutralization, the rate of gel formation is time and temperature dependent. Gelling will occur slowly at 4-8°C, and more rapidly when warmed to 37°C. For this reason it is imperative to store collagen-1 stocks and working solutions at 4-8°C at all times to avoid unwanted gelling. It is easier to handle low volume

(<500µL) collagen-1 solutions using pre-cooled (from a fridge), wide bore pipette tips.

We recommend sourcing a recently manufactured lot of collagen-1 (<3 months old).

Day 1 – Coat the ASSAY PLATE with a thin layer of collagen-1 (10min, wait 1d)

1. In a 15 ml Falcon tube, dilute collagen-1 stock to 300 µg ml⁻¹ in 20 mM acetic acid by slowly pipetting 360 µl of 5 mg ml⁻¹ collagen-1 into 5.64 ml acetic acid. Pipette five times to mix, ensuring any residual collagen-1 in the pipette tip has been washed into the vial.
2. Dispense 50 µl of diluted collagen-1 into each well of a 96-well ImageLock™ plate. Gently rock the plate to ensure even coating of each well. Place the plate in a 37 °C incubator, 5% CO₂ overnight.

Detailed Scratch Wound Cell Invasion Protocol: Collagen-1 + HT1080 cells

DAY 2 – Seed cells onto the ASSAY PLATE (30min, wait 4h)

1. Remove a freshly confluent T75 flask of HT1080 cells from the cell incubator and place within a sterile cell culture hood.
2. Remove media, and wash cells with D-PBS (10ml).
3. Add 1ml Trypsin/EDTA to detach cells, leave for 2-3min.
4. Add 9ml of culture media, and re-suspend cells using a 10ml serological pipette.
5. Perform a cell count (e.g. trypan blue staining + haemocytometer).
6. Centrifuge the cell suspension (185 xg, 4 min) and re-suspend the cell pellet in culture media at 2.5×10^5 cells ml^{-1} .
7. Remove ASSAY PLATE from the incubator and wash twice with room temperature D-PBS 100 μl per well).
8. Plate cells (100 μl per well, i.e. 25K cells) into every well of the ASSAY PLATE. Do not leave any empty (dry) wells - these will damage the WoundMaker™.
9. Allow the cell plate to sit at room temperature for 5min to allow cells to evenly disperse across the bottom of the plate. Place the lidded plate into a 37°C incubator, 5% CO₂ **for 4h**.

Day 2 – Prepare REAGENT plate & NEUTRALIZATION plate (30min)

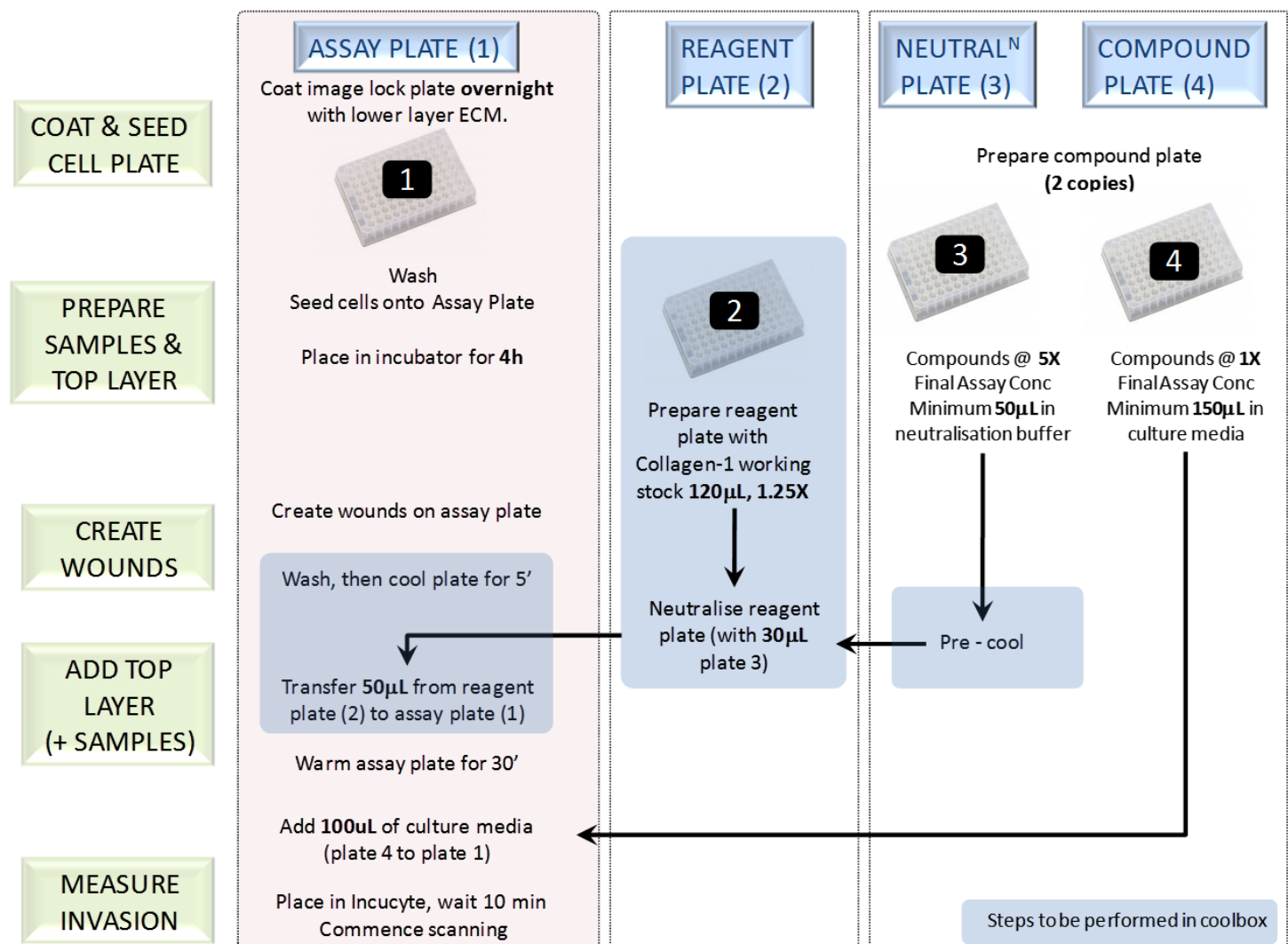
1. Place 2 standard 96-well plates in the pre-chilled CoolBoxes 96F at 4 - 8 °C for 5 min. Keep these plate on the CoolBox throughout the protocol.
2. REAGENT PLATE: add 200 μL collagen-1 working stock to each well of columns 1 & 4 (for Invasion wells).
3. REAGENT PLATE: add 200 μL of 20mM Acetic Acid to each well of columns 7 & 10 (for Migration controls).
4. NEUTRALIZATION PLATE: add 300 μL of 5x neutralization buffer to each well of column 1.
5. Begin neutralizing the collagen-1/acetic acid containing wells by adding (with a multichannel pipette) 50 μL from column 1 of the NEUTRALIZATION PLATE to column 1, 4, 7 and 10 of the REAGENT PLATE.
6. Mix in the buffer by pipetting five times (100 μl per well) in the lower corner of each well followed by five times in the opposite bottom corner of each well. Inspect wells to ensure they are an even yellow to orange color, indicating complete neutralization.
7. Remove bubbles by gently squeezing a wash bottle (containing 100% ethanol with the inner straw removed) to blow vapor over the surface of each well. Try to keep the tip of the wash bottle approximately 5cm from the media surface.
8. **NOTE:** in sample testing experiments, the NEUTRALIZATION PLATE should contain samples prepared (diluted) in neutralization buffer, at 5X the final assay concentration. A second plate containing compounds (the COMPOUND PLATE (prepared in media at 1X) will also need to be prepared (see Figure).

DAY2 – Wound, add top layer & schedule scans (40 – 50min)

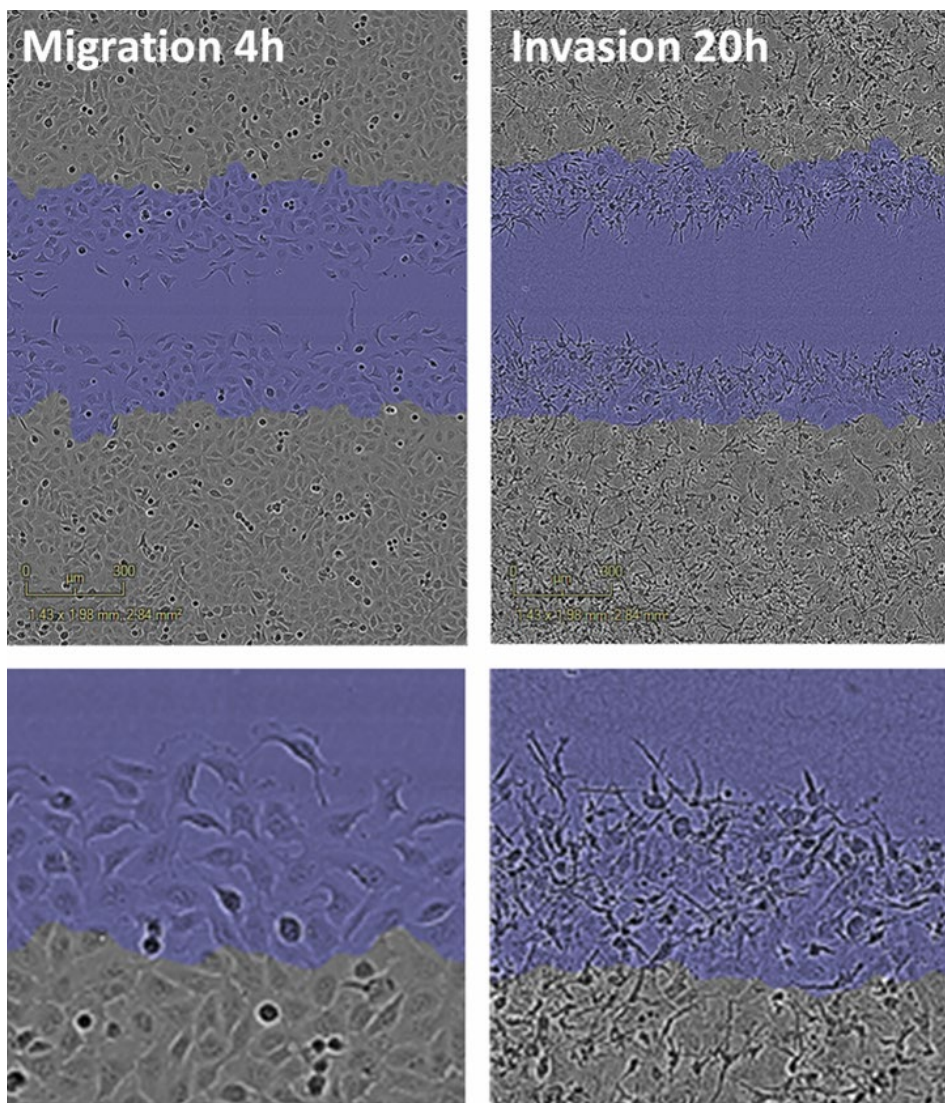
1. Carefully remove the ASSAY plate from the incubator.
2. Follow the 96-well WoundMaker™ procedure to simultaneously create wounds in all wells. Visually inspect wounds with an inverted microscope, to ensure that you have correctly used the WoundMaker™.
3. Immediately (and carefully) wash the cells twice with cell culture media. After the final wash, add culture media (100 μl) to each well and place plate in a pre-chilled CoolBox 96F to equilibrate for 5 min. Remove and discard (cool) media from the ASSAY PLATE.
4. Transfer neutralized solutions from the REAGENT PLATE to the ASSAY PLATE (50 μl per well: Col 1 Reagent plate to cols 1,2,3 on assay plate, Col 4 Reagent plate to cols 4,5,6 on assay plate etc). Remove bubbles as before.
5. Place the ASSAY PLATE on to the pre-warmed CoolSink 96F in a 37 °C, 5% CO₂ incubator for 30 min.
6. Add 100 μl of additional culture media to each well of the ASSAY plate. Do not mix. Remove bubbles. Place the de-bubbled cell plate in the IncuCyte, close the drawer and incubator door.
7. Allow the cell plate to equilibrate in the IncuCyte for 10min prior to scheduling the first scan – this will reduce the likelihood of condensation on the lower side of the plate which can hinder image quality.
8. In the IncuCyte ZOOM® software, schedule repeat scanning for every 3h for 48h, with the first scan to commence immediately. Set the assay plate to scan one image per well (Wide Mode if available) and ensure 'scan type' is set to Scratch Wound.
9. Wash and store the WoundMaker™ according to the established Wash Protocols.

Detailed Scratch Wound Cell Invasion Protocol: Collagen-1 + HT1080 cells

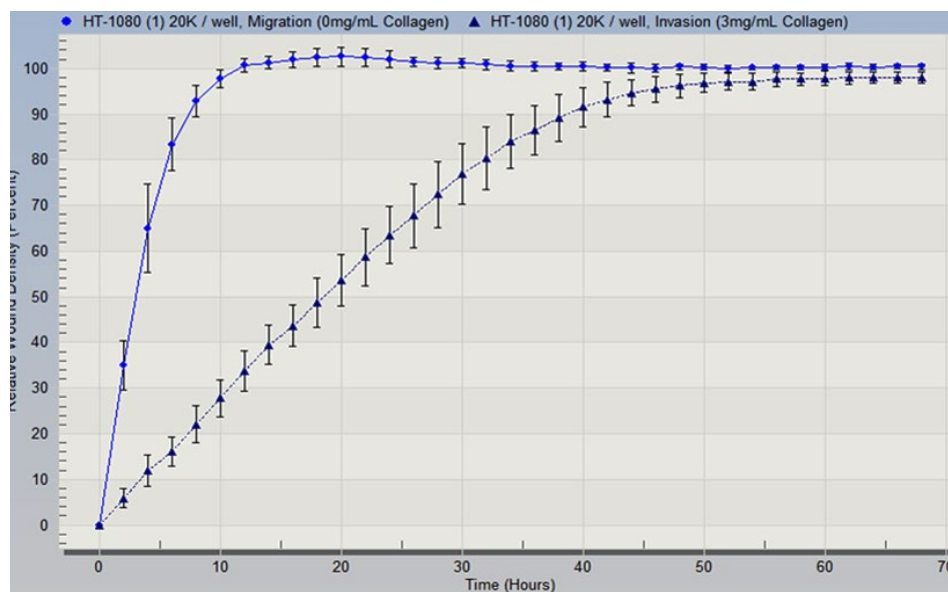
Schematic diagram of Collagen-1 Invasion Assay Workflow with test samples



Detailed Scratch Wound Cell Invasion Protocol: Collagen-1 + HT1080 cells



Representative phase contrast images of HT1080 cells (upper; 10X magnification, lower; digitally zoomed) taken at 4h post wounding for migration (left) and 20h post wounding for invasion (right). Note, clean wound boundaries and symmetrical migration/invasion. Also note the characteristic differences in cell morphology.



Summary data time courses of migration and invasion (% relative wound density; points are mean values and vertical bars show standard deviation).

Recommended Reading

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